Thermal Denaturation of Beef Cardiac Troponin and Its Subunits with and without Calcium Ion†

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ABSTRACT: Differential scanning calorimetry has been used to study the thermal denaturation of beef cardiac troponin (Tn), the bimolecular complexes of troponin subunits, and the individual subunits at pH 7.0 in 0.6 M KCl and 10 mM $MgCl_2$. Measurements were made in both the presence and absence of 10 mM $CaCl_2$. The denaturation temperature (T at which the protein is 50% denatured), the enthalpy and entropy of thermal denaturation, and the heat capacity differences between native and denatured forms have been determined. With 10 mM $CaCl_2$ present, all denaturations are reversible. In the absence of Ca^{2+}, all denaturations except for the troponin and its TC subunit are reversible. The relative order of stability to thermal degradation has been determined from the denaturation temperature. The TnC subunit is the least thermally stable protein in the absence of Ca^{2+} and the most stable in the presence of Ca^{2+}. Complex formation in the absence of Ca^{2+} of TnC with TnI and TnT increases thermal stability relative to TnC but decreases stability relative to TnI or TnT. Complex formation in the presence of Ca^{2+} results in the opposite behavior. While the increase in T_d with Ca^{2+} is large, the presence or absence of Ca^{2+} has no effect on the enthalpy of denaturation for troponin or its C subunit. For reversible systems, standard free energies, enthalpies, and entropies of denaturation were calculated as a function of T. $\Delta H°$ is positive and increases with temperature. $\Delta S°$ is also positive and increases with temperature. The positive $\Delta S°$ is in keeping with the idea that bound solvent is released on denaturation. The enthalpy and entropy act in opposing directions in determining the free energy of denaturation. Addition of Ca^{2+} to TnC (or TnCI) causes a decrease in both $\Delta S°$ and $\Delta H°$. The net result of Ca^{2+} is to increase $\Delta G°$ (i.e., increase stabilization). The thermodynamic data also suggest that stabilization of troponin by mutual interaction of all three subunits occurs.

The interaction of troponin (Tn)[1] or its C subunit with Ca^{2+} and the effect of association between the TnI, TnC, and TnT subunits of Tn have been extensively studied with a number of physical techniques (NMR, CD, fluorescence, etc.). However, there is very little thermodynamic data available for the Tn system. The binding of Ca^{2+} to skeletal TnC has been studied by Potter et al. (1977) and Yamada (1978). The results of these studies are not in good agreement. However, both workers agree that both enthalpy and entropy changes act in concert to produce a large negative free energy change. Release of bound water has been suggested to be the cause of the increase in entropy on Ca^{2+} binding since it was known that Ca^{2+} binding to skeletal TnC caused an increase in α-helical structure in TnC (Kawasaki & van Eerd, 1972; Potter et al., 1976). In the skeletal system, the enthalpy changes on adding Ca^{2+} were smaller for TnC than for Tn (Yamada et al., 1976). While there are numerous similarities between the properties of cardiac and skeletal Tn, there are a number of major differences between the two systems. Both systems contain two Ca^{2+}–Mg^{2+} high affinity binding sites (Potter & Gergely, 1975; Leavis & Kraft, 1978) and two Mg^{2+}-specific sites (Potter & Gergely, 1975; Kohama, 1979). However, the cardiac system appears to have only one Ca^{2+}-specific regulatory site (Burtnick & Kay, 1977; Potter, 1977; Leavis & Kraft, 1978), while the skeletal system had two such sites (Potter & Gergely, 1975). Differences in binding constants for Ca^{2+} have also been shown (Hincke et al., 1978). Differences in amino acid sequence have also been reported (Collins et al., 1973; van Eerd & Takahashi, 1975). Considering these differences, there is no reason to predict that the thermodynamics of Ca^{2+} binding would be the same for cardiac and skeletal Tn.

Differential scanning calorimetry can measure the enthalpy of denaturation (ΔH_{cal}), the change in heat capacity between native and denatured states (ΔC_p^d), and, if the system is reversible, the entropy of denaturation. A measure of the thermal stability of the proteins can also be obtained by comparisons of denaturation temperatures (T_d, the temperature at which the protein is 50% denatured). If the denaturations are reversible, the standard thermodynamic parameters for denaturation at any T can be calculated. In our measurements of the Tn system, 0.6 M KCl and 10 mM $MgCl_2$ at pH 7.0 was chosen as the solvent, since solubility of TnI and TnT requires high salt. Mg^{2+} was included since it is generally believed that the Ca^{2+}–Mg^{2+} sites are occupied by Mg^{2+} during relaxation.

Materials and Methods

Ultrapure $CaCl_2$, $(NH_4)_2SO_4$, HCl, and urea were used. All other chemicals were of reagent grade. The KCl (as a 4 M solution) was passed through Chelex 100 resin prior to use. DEAE-Sepharose CL-6B, SP-Sephadex C-50, and Sephadex G-75 were obtained from Pharmacia. Double-deionized water was used throughout.

The protein molecular weights were taken to be 72000 for

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[1] Abbreviations used: Tn, troponin; TnT, TnC, and TnI, the T, I, and C subunits of troponin, respectively; TnTC and TnIC, the bimolecular complexes of the T and C and the I and C subunits of troponin, respectively; the superscripts + and − indicate the presence or absence of 10.0 mM $CaCl_2$ in the solutions; MOPS, 3-(*N*-ethylmorpholino)-propanesulfonic acid; DSC, differential scanning calorimetry; T_d, temperature at which the protein is 50% denatured; ΔH_{cal}, enthalpy of denaturation obtained from DSC; ΔS_{T_d}, enthalpy of denaturation at T_d.

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troponin and 30 500, 18 500, and 23 000 for the T, C, and I units, respectively. The buffer solution for all calorimetric measurements consisted of 0.6 M KCl, 50 mM MOPS, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. Solid $CaCl_2$ was added to the samples for the measurements with 10 mM Ca^{2+}.

Fresh beef hearts were chilled, dissected free of excess fat, and cut into 1-in. cubes for homogenization. Troponin was prepared by the method of Tsukui & Ebashi (1973) as modified by Burtnick (1977). The major modifications were the omission of the 0.2 M LiCl wash (the yield decreased drastically if this was included), the substitution of centrifugation for cheesecloth during the washes (TnT yield reduced if cheesecloth use), and the inclusion of 0.1 mM phenylmethanesulfonyl fluoride and 0.5 mM dithiothreitol in the initial homogenization. The fractions from the DEAE-Sepharose CL-6B column were pooled, and the protein was precipitated with $(NH_4)_2SO_4$. The pellet was resuspended in a small volume of buffer and exhaustively dialyzed against the same buffer (3 days with frequent changes of buffer).

The subunits of bovine cardiac troponin were separated and purified by the methods of Brekke & Greaser (1976). The isolated subunits were dialyzed exhaustively against buffer. The protein concentrations were calculated from the A_{277nm} values of Hincke et al. (1978).

The reconstituted troponin and the bimolecular complexes were prepared by mixing the protein solutions on a 1:1:1 or 1:1 molar ratios. The sample was then passed through a Sephadex G-75 column equilibrated with buffer plus 10 mM ethylene glycol bis(β-aminoethyl) ether)-*N,N,N',N'*-tetraacetic acid (EGTA). Recovery was 65–80%.

The protein solutions were concentrated by dialysis against dry Ficol 400, followed by dialysis against the buffer. Protein concentration ranged between 70 and 110 mg/mL. The proteins were stored in 75-μL lots at −20 °C. The purity of the various subunits was checked on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate. A slight modification of the method of Weber & Osborn (1969) was used. [The protein was dissolved in 4 M urea and 10 mM (ethylenedinitrilo)tetraacetic acid (EDTA).] Low protein loadings (10 μg) were used to check molecular weight, and high protein loadings (100 μg) for purity. No additional protein bands were observed with high loading.

Endogenous Ca^{2+} in the solutions was measured by atomic absorption spectroscopy. In the solutions without added Ca^{2+}, the [Ca^{2+}] ranged between 0.2 and 0.4 ppm. Hence, in these concentrated troponin solutions, no more than 0.5% of the high affinity Ca^{2+} binding sites were occupied by Ca^{2+}. In the troponin C solutions without added Ca^{2+}, occupancy of Ca^{2+} binding sites by Ca^{2+} was 0.1%.

Thermal measurements were made by using a Perkin-Elmer differential scanning calorimeter calibrated with indium. Samples (70 μL) were pipetted into 75-μL stainless steel pans with a Hamilton syringe. The reference pan consisted of a buffer blank whose volume was adjusted to the volume of buffer in the sample pan [volume in reference = volume in sample − (milligrams of protein in sample × partial specific volume)]. The value 0.73 was used for the partial specific volume of troponin and its subunits (Byers et al., 1979). The heating rates were 2.5, 1.25, and 0.63 °C min^{-1}. A solvent blank was run before and after each experiment. For each protein, at each heating rate, 3–5 samples were run. When the reversibility of the denaturations was checked the samples were cooled at the same rate at which they were initially heated. They were then reheated at this rate.

Examples of calculations are given in Figure 1. First, the base line was subtracted from the thermogram, and then ΔH_{cal} was obtained from the area under the thermogram. The area was measured with a planemeter. T_d was taken from the temperature at which the protein was 50% denatured, i.e., the T at which the peak area was divided in half. ΔC_p^d, the difference in heat capacity between native and denatured forms, was obtained by extension of C_p for native and denatured forms at T_d.

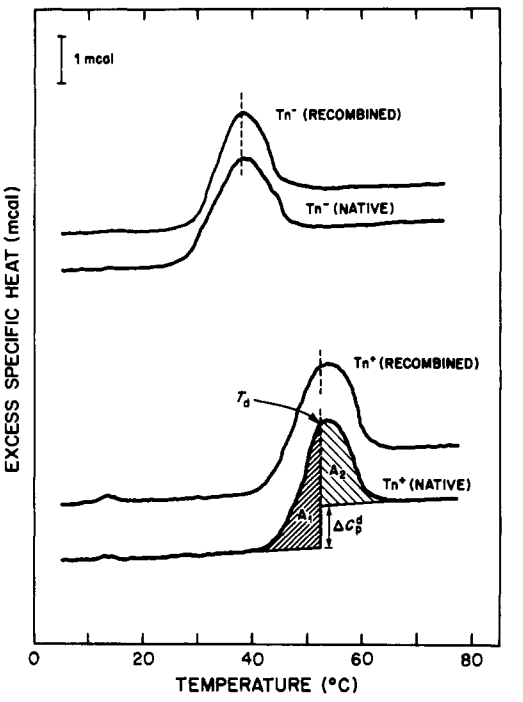

FIGURE 1: DSC scans for native and synthetic troponin. All samples were run at 2.5 °C min^{-1} in 0.6 M KCl, 10.0 mM $MgCl_2$, 50 mM MOPS, and 10.0 mM 2-mercaptoethanol at pH 7.0. The + indicates addition of 10.0 mM $CaCl_2$, and − indicates no $CaCl_2$ added. The concentrations were 74.3 and 81.9 mg/mL, respectively, for reconstituted and native troponin in the absence of Ca^{2+} and 80.6 and 68.1 mg/mL, respectively, in the presence of Ca^{2+}.

Results

Typical DSC scans for troponin are shown in Figure 1 (along with the methods of calculation of ΔH_{cal}, T_d, and ΔC_p^d). With or without Ca^{2+}, there is no observable difference between the reconstituted and natural troponin. The enthalpy, denaturation temperature, and heat capacity data in Table I also show that there is no difference between the natural and the reconstituted troponin. From the calorimetric data, it appears that the presence of urea is not required for reconstitution of troponin. This is in agreement with the results of C. M. Kay and co-workers (personal communication).

The thermal denaturation of troponin in the presence of calcium (Tn^+) is totally reversible. Only one transition is observed, which suggests that the denaturation is highly cooperative. With both 10 mM $MgCl_2$ and 10 mM $CaCl_2$ present, all divalent ion binding sites on Tn should be occupied. In these experiments, the Tn concentration varied between 1.0 and 1.4 mM. However, in the absence of calcium (Tn^-), the denaturation is only 41% reversible. Under these conditions, both the Mg^{2+}-specific and the Ca^{2+}–Mg^{2+} binding sites should be occupied by Mg^{2+}. For Tn^-, one major transition and a small shoulder are observed. While calcium increases the transition temperature by 14 °C, there is no significant change in ΔH_{cal} or in ΔC_p^d.

Typical DSC scans for the individual subunits are shown in Figure 2. The thermal denaturation of all subunits and bimolecular complexes is completely reversible except for

Table I: Calorimetric Data for Troponin and Its Subunits in 0.6 M KCl, 50 mM MOPS, 10 mM $MgCl_2$, and 10 mM 2-Mercaptoethanol at pH 7.0

sample	±Ca (10 mM)	T_d° (°C ± 0.2)	ΔH_{cal} (kcal mol^{-1})	ΔC_p[d] (kcal K^{-1} mol^{-1})	ΔS_{T_d} (kcal mol^{-1})
natural troponin	−	34.4	134 ± 2	0.9 ± 0.3	
reconstituted troponin	−	34.3	134 ± 2	0.9 ± 0.2	
natural troponin	+	48.3	140 ± 4	1.0 ± 0.2	0.436 ± 0.012
reconstituted troponin	+	48.3	138 ± 2	0.9 ± 0.3	0.430 ± 0.006
troponin C	−	32.2	44 ± 1	0.6 ± 0.2	0.144 ± 0.007
troponin C	+	49.5	43 ± 1	0.6 ± 0.2	0.133 ± 0.003
troponin I	−	47.3	40 ± 1	0.9 ± 0.2	0.125 ± 0.003
troponin T	−	39.1	35 ± 1	0.6 ± 0.2	0.112 ± 0.003
troponin CI	−	38.3	79 ± 1	0.8 ± 0.2	0.254 ± 0.003
troponin CI	+	49.3	84 ± 2	0.8 ± 0.2	0.261 ± 0.006
troponin TC	−	35.2	83 ± 2	0.8 ± 0.2	
troponin TC	+	44.3	83 ± 2	0.7 ± 0.3	0.261 ± 0.007

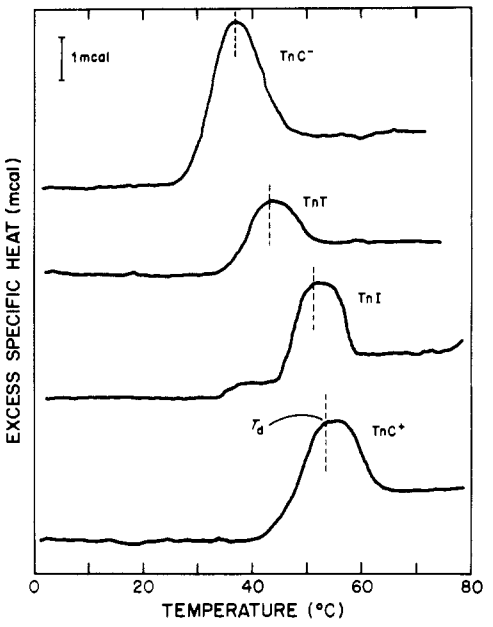

FIGURE 2: DSC scans for the C, I, and T subunits of troponin. Experimental conditions indicated in Figure 1. The concentration of the TnC in absence of Ca^{2+} was 98.3 and in presence of Ca^{2+}, 108.0 mg/mL. The concentrations of the TnT and TnI were 73.4 and 87.0 mg/mL, respectively.

$TnTC^-$. The ΔH_{cal} for $TnTC^-$ after the sample is reheated is only 27% of the original value. The transitions are symmetrical for all subunits (including $TnTC^\pm$ and $TnCI^\pm$ which are not shown) except for TnI. For TnI, a small but reproducible change in C_p occurs before the main transition. This change represents about 12% of the total ΔH_{cal} and also appears in the reheated samples. This type of change has been observed in other protein systems and has been attributed to dissociation of aggregates (Potekhin & Privalov, 1978). Since TnI is known to aggregate (Greaser & Gergely, 1973), disaggregation is a reasonable assumption to explain the thermogram for TnI. There is no indication of any small transition before the main transition with TnCI or Tn. This suggests that the state of aggregation in TnI is altered by binding of the I component to TnC.

At these high protein concentrations (70–100 mg/mL), aggregation might be expected to occur in all solutions. There is no evidence for aggregation in all thermograms except for TnI. Aggregation at lower concentrations has been reported for TnT (Greaser & Gergely, 1973). It is possible that the higher KCl concentration used in this study (0.6 M) and/or the presence of Mg^{2+} inhibits aggregation. Aggregation has also been reported for TnC when the concentration of free Ca^{2+} approaches 10^{-4} M (Murray & Kay, 1972). In addition to possible changes in C_p in the thermograms before the main transition, aggregation should also increase ΔH_{cal}. In our samples, there is no indication of aggregation for TnC^+ in the thermogram nor is there an increase in ΔH_{cal} on addition of Ca^{2+} to TnC (Table I). With 10 mM $CaCl_2$ present and 70–100 mg/mL protein (4.3–6.0 mM), the concentration of free Ca^{2+} is less than 10^{-4} M if the binding constant for Ca^{2+} to TnC is 2×10^7 (Hincke et al., 1978). It is possible that disaggregation and denaturation of the protein occur simultaneously. Since there is no change in ΔH_{cal} on addition of Ca^{2+} to TnC, either there is no significant change in aggregation on addition of Ca^{2+} or the contribution of aggregation to ΔH_{cal} is small. There are no data on the troponin system for comparison. Tsong et al. (1970) found no change in ΔH_{cal} for ribonuclease between 1 and 27 mg/mL, and Jacobson & Turner (1980) found similar results between 50 and 100 mg/mL. It is likely that contributions to ΔH_{cal} and the importance of aggregation at high protein concentration depend on the nature of the particular protein studied.

The results from the DSC are summarized in Table I. T_d is a function of heating rate. The T_d° values shown in Table I were obtained by a linear extrapolation of T_d at 2.5, 1.25, and 0.63 °C min^{-1} heating rates to 0 heating rate. The correlation coefficient in this extrapolation ranged from 0.97 to 1.00. The largest change with heating rate was found in TnC^+. In this case, T_d decreased from 53.7 °C at 2.5 °C min^{-1} to 52.0 °C at 1.25 °C min^{-1} and to 50.4 °C at 0.63 °C min^{-1}. In all cases where the TnC unit is involved (either alone or as part of a complex), addition of 10 mM $CaCl_2$ produces a large increase in T_d°. If T_d is used as a relative measure of thermal stability, TnC^- is the least stable and TnC^+ is the most stable protein. Addition of any subunit to TnC^- produces a complex that is more stable that TnC^- but less stable than the subunit which is added. Tn^- is only slightly more stable than TnC^- (~2 °C). The thermal stabilities of TnC^+ and $TnCI^+$ are equal. $TnTC^+$ is less stable than either TnC^+ or Tn^+. Tn^+ is slightly less stable than TnC^+. There are relatively few calorimetric studies of protein–protein interaction. For trypsin with soybean trypsin inhibitor or with ovomucoid (Donovan & Beardslee, 1975), the T_d for the complex was greater than for either component. The Tn subunit interactions appear to produce a complex in which the thermal stability is intermediate between the components rather than greater than either component.

ΔH_{cal} is not dependent on heating rate. The values reported in Table I are an average of 11 determinations (five at 2.5 °C min^{-1} and three each at 1.25 and 0.63 °C min^{-1} heating rates). For Tn, TnC, and TnTC there is no change in ΔH_{cal} on addition of calcium. For TnCI there is a small but significant

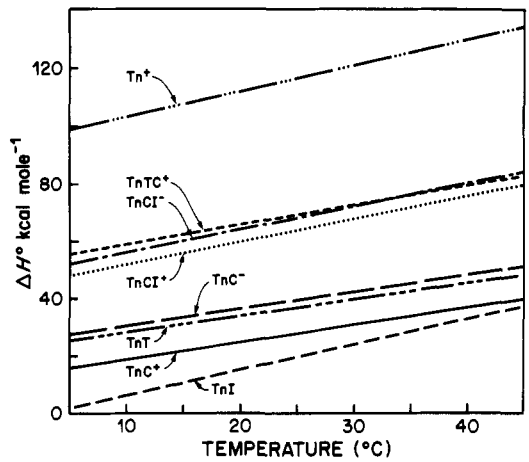

FIGURE 3: Standard enthalpy changes for the denaturation of Tn and its subunits as a function of T. Solvent and pH are given in Figure 1. $\Delta H°$ values were calculated from ΔH_{cal} and ΔC_p^d by assuming ΔC_p^d independent of T. + indicates the presence of 10.0 mM Ca^{2+} and – indicates no Ca^{2+} added. Tn^+(—··—); $TnTC^+$ (---); $TnCI^-$ (—·—); $TnCI^+$ (···); TnC^- (— —); TnC^+ (—); TnT (—·--); TnI (– –).

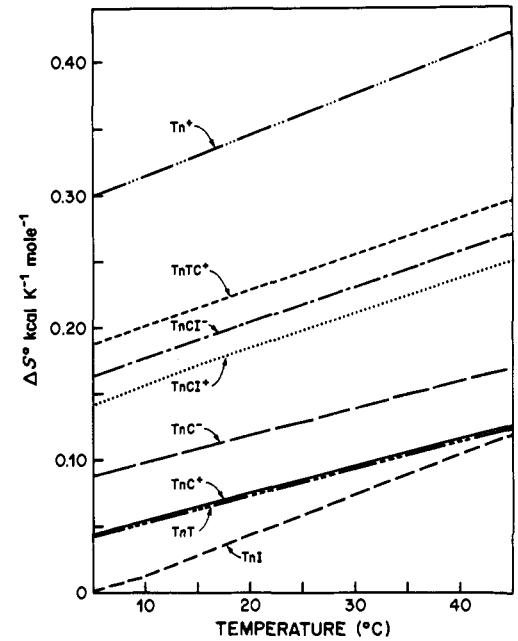

FIGURE 4: Standard entropy changes for the denaturation of Tn and its subunits as a function of T. Conditions and symbols same as in Figure 3.

(to a 1% probability) increase in ΔH_{cal} on addition of calcium. ΔH_{cal} values of $TnTC^{\pm}$ and $TnCI^{\pm}$ are close to the sum of ΔH_{cal} for their components. However, ΔH_{cal} for $Tn^{\pm}$ is considerably greater than the sum of the individual components. This suggests that there is a higher degree of protein–protein interaction in the three unit complex, but this can only be regarded as a suggestion since the T_d values of the complex and the components are different.

ΔC_p^d values are also independent of heating rate. The ΔC_p^d values shown in Table I are the average of the 11 determinations. The standard deviations are relatively high due to the inherent difficulties of obtaining data by base-line subtraction and extrapolation of C_p to T_d. There is no change in ΔC_p^d on addition of calcium.

The entropy of the thermal denaturation at T_d was calculated for the reversible denaturations by assuming that the system was at equilibrium and that the free energy change at the midpoint of the transition was zero. The area under the thermogram was assumed to be proportional to the amount of protein denatured. The protein was assumed to be half-denatured at T_d and $\Delta H_{cal} = T_d \Delta S_{Td}$ [reviewed by Biltonen & Freire (1978)]. These values are also given in Table I. The entropies of denaturation of $TnTC^+$ and $TnCI^{\pm}$ are close to the value for the sum of their individual components, but the entropy of Tn^+ is considerably higher than the sum of the three components. This suggests that at T_d a higher degree of order is found in Tn^+ than in the bimolecular complexes.

ΔH_{eff} can be calculated from all thermograms by the method of Privalov & Khechinashvili (1974). For all reversible transitions except Tn^+, the ratio of $\Delta H_{cal}/\Delta H_{eff}$ is 1.00 within experimental error. This ratio suggests that a two-state model for denaturation may be appropriate and that the denaturations are highly cooperative. A significant proportion of intermediate states is indicated for Tn^+ ($\Delta H_{cal}/\Delta H_{eff}$ 1.14).

For the reversible denaturations, the enthalpy ($\Delta H°$), the entropy ($\Delta S°$), and the free energy change ($\Delta G°$) were calculated as a function of temperature from the Gibbs equation and the ΔH_{cal}, $T_d°$, and ΔC_p^d values obtained from the thermograms. ΔC_p^d was assumed to be independent of temperature. In Figure 3, $\Delta H°$ is shown as a function of temperature. $\Delta H°$ is positive and increases with temperature for troponin and all of its subunits. The order of $\Delta H°$ values at constant temperature is individual subunits < bimolecular complexes < Tn^+. Comparisons at constant temperature show that addition of Ca^{2+} decreases $\Delta H°$.

In Figure 4, $\Delta S°$ is shown as a function of temperature. $\Delta S°$ is positive and increases with temperature. A decrease in $\Delta S°$ with temperature might be predicted from a consideration of possible effects of temperature on the native and denatured states of the proteins. Less ordered protein arrangements should be produced at high T, and the effect on the native state (which is more ordered and stabilized by noncovalent forces) should be greater than the effect on the already disordered denatured state. However, $\Delta S°$ measures the total entropy change for the denaturation, and changes in the number of H_2O molecules bound could account for the increase in $\Delta S°$ with temperature. At constant temperature, the order of $\Delta S°$ values is individual subunits < bimolecular complexes < Tn^+. Comparisons at constant temperature also show that Ca^{2+} decreases $\Delta S°$; i.e., in terms of entropy, a more ordered protein system is formed in the presence of Ca^{2+}. If Ca^{2+} binds and causes a conformational change, i.e., coil–helix transition which has been suggested by CD (van Eerd & Kawasaki, 1972) and NMR measurements (Seamon et al., 1977), such an entropy decrease would be expected.

The entropy and enthalpy changes in the troponin system are in the opposite direction. This compensatory behavior has been found in a number of systems. The decrease in $\Delta G°$ with temperature (Figure 5) indicates that the $T\Delta S°$ term is large enough to overcome the $\Delta H°$ term in calculating $\Delta G°$. The overall effect at constant temperature is that Ca^{2+} increases $\Delta G°$. Hence, the protein systems containing the C subunit are more stable in the presence of Ca^{2+}.

Since the slope of the plot of $\Delta G°$ vs. T is $-\Delta S°$ (which is itself a function of T), the order of stability of Tn^+ and its various components as measured by the magnitude of $\Delta G°$ also varies with temperature. At any constant T (<45 °C), Tn^+ is more stable than any of its subunits. At lower temperatures, TnT is the least stable component, while at higher T, TnC^- is the least stable component. TnT has been reported to be the least stable component of Tn (Drabikowski et al., 1971; Dabrowska et al., 1973) in terms of enzymatic degradation. This result may vary with temperature. The relative order of stability at any constant temperature (as indicated by $\Delta G°$) is not the same as the relative order of stability to thermal denaturation where T is the variable (T_d).

Table II: Thermodynamic Parameters for Tn and Its Subunits ±10.0 mM Ca^{2+} in 0.6 M KCl, 10.0 mM $MgCl_2$, and 10 mM 2-Mercaptoethanol at pH 7.0[a]

	25 °C			37 °C		
	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (kcal K^{-1} mol^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (kcal K^{-1} mol^{-1})
Tn^+	9.2 ± 0.3	117 ± 7	0.36 ± 0.02	4.7 ± 0.1	128 ± 3	0.40 ± 0.01
$TnCI^+$	5.6 ± 0.2	64 ± 5	0.20 ± 0.02	3.0 ± 0.1	74 ± 1	0.23 ± 0.01
$TnCI^-$	3.1 ± 0.1	68 ± 3	0.22 ± 0.01	0.3 ± 0.1	78 ± 1	0.25 ± 0.01
$TnTC^+$	4.6 ± 0.1	69 ± 4	0.22 ± 0.01	1.8 ± 0.1	78 ± 1	0.24 ± 0.01
TnC^+	2.7 ± 0.2	28 ± 5	0.08 ± 0.02	1.5 ± 0.1	35 ± 2	0.11 ± 0.01
TnC^-	1.0 ± 0.1	40 ± 3	0.13 ± 0.01	−0.7 ± 0.1	47 ± 1	0.15 ± 0.01
TnI	2.1 ± 0.2	20 ± 4	0.06 ± 0.01	1.1 ± 0.1	31 ± 2	0.09 ± 0.01
TnT	1.4 ± 0.1	27 ± 2	0.08 ± 0.01	0.2 ± 0.1	34 ± 2	0.11 ± 0.01

[a] Plus indicates the presence of 10 mM Ca^{2+}, and minus indicates that no Ca^{2+} was added. The standard deviations were calculated at each temperature from $(\Sigma d_i^2)^{1/2}$, where d_i was the deviation produced at the temperature indicated by variations equal to the standard deviations experimentally determined in ΔH_{cal}, $T_d°$, and ΔC_p^d, respectively.

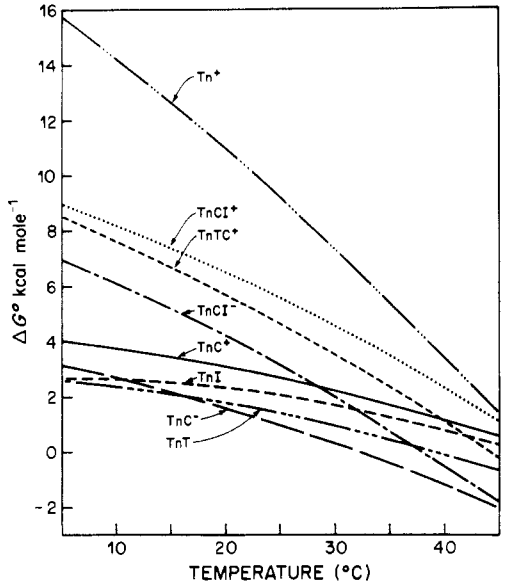

FIGURE 5: Standard free energy changes for the denaturation of Tn and its subunits as a function of T. Conditions and symbols same as in Figure 3.

Figures 3–5 are useful for showing trends in the variation of the thermodynamic parameters with T. However, with the eight protein systems shown in these figures, it is difficult to indicate error limits. Since the error in ΔC_p^d is relatively large, the standard deviations in $\Delta H°$, $\Delta S°$, and $\Delta G°$ could be significant and limit the interpretation of data. The calculated thermodynamic parameters at 25 and 37 °C are shown in Table II along with their standard deviations. The deviations in $\Delta H°$, $\Delta S°$, and $\Delta G°$ values were calculated by assuming the standard deviations in Table I. The values reported in Table II as standard deviations were obtained from the square root of the sum of the squares of the deviations in the thermodynamic values caused by the individual variation in ΔH_{cal}, ΔC_p^d, and $T_d°$. The standard deviation in ΔC_p^d is the largest component in the standard deviations reported in Table II. Standard deviations of each sample increase as the difference between T_d and T is increased. The effect of the assumption that C_p^d is independent of temperature has not been calculated. The deviations caused by this assumption should also decrease as T approached T_d. Hence, the thermodynamic parameters are also reported at 37 °C (which is close to physiological temperature and closer to the T_d values of the various protein systems). Table II shows that the increase in $\Delta G°$ due to Ca^{2+} addition is significant. The decrease in $\Delta H°$ due to Ca^{2+} is small but significant for TnC, but not for TnCI, at 25 °C. The change in $\Delta H°$ due to Ca^{2+} is small but significant at 37 °C. The Ca^{2+} effect on $\Delta S°$ is significant for TnC but not for TnCI.

The effect of protein–protein interaction at constant temperature can be shown by comparisons of the $\Delta G°$, $\Delta H°$, and $\Delta S°$ values in Table II. If there were no effect on $\Delta G°$, $\Delta H°$, and $\Delta S°$ due to protein–protein interaction, the values for the complexes should be equal to the sum of the values for the individual components. The differences between these sums and the calculated values are a measure of the effect of protein–protein association. At 25 °C, the stabilization due to the addition of TnC^+ to TnI or TnT is small; i.e., [$\Delta G°$(complex) − $\Delta G°$(components)] = 0.8 and 0.5 kcal mol^{-1} for $TnCI^+$ and $TnTC^+$, respectively. There is no stabilization found for addition of TnC^- to TnI. Only if the whole complex is considered is stabilization significant (i.e., 3.0 kcal mol^{-1}). The stabilizing effects of a three unit complex are also apparent if TnT were added to $TnCI^+$ [$\Delta(\Delta G°)$ = 2.2 kcal mol^{-1} at 25 °C] and if TnI were added to $TnTC^+$ [$\Delta(\Delta G°)$ = 2.5 kcal mol^{-1} at 25 °C]. The changes in $\Delta H°$ and $\Delta S°$ are also larger for the trimolecular unit compared to the bimolecular complexes. The entropy changes indicate that there is a considerably higher degree of order in the trimolecular complex than in the bimolecular units. Similar results are obtained if the comparisons are at 37 °C.

Discussion

Only one thermal transition has been observed with a number of simple globular proteins [reviewed by Biltonin & Freire (1978)]. However, there have been a number of cases with monomeric proteins [as examples, fibrinogen by Donovan & Mikalyi (1974), $tRNA^{Val}$ by Brandts et al. (1974), tropomyosin by Potekhin and Privalov (1978), and myosin by Potekhin et al. (1979)] where differential melting of various regions of the protein have been observed. The differences in thermal stability between the individual subunits of Tn is large (T_d = 32 °C for TnC compare to 39 °C for TnT and 47 °C for TnI). Differential melting regions in the Tn complexes would not have been surprising. However, only one transition was observed. It appears that interactions between the subunits in the Tn system drastically alter ther thermal stability of the individual subunits. There are only a few complex protein systems which have been studied. Only one thermal transition has been observed for trypsin with soybean trypsin inhibitor or ovomuccoid (Donovan & Beardslee, 1975) and for lactic dehydrogenase (Jacobson & Braun, 1977). It appears that when the interaction between subunits is strong dissociation and denaturation occur simultaneously and only

Table III: Thermodynamic Parameters Associated with Binding of Ca^{2+} to TnC[a]

	cardiac TnC		skeletal TnC				
			Yamada (1978)[b]		Potter et al. (1977)		
KCl (M)	0.6		0.1		0.1		
$MgCl_2$ (mM)	10.0		1.0				
pH	7.0		8.8		7.0		
temp (°C)	25	37	10	10	25	25	25
	$i=2$	$i=2$	$i=2$	$i=3$	$i=2$	$i=3$	$i=4$
ΔG°_i (kcal mol^{-1})	−1.7 ± 0.2	−2.2 ± 0.1	−9.1	−15.6	−24.2	−34.3	−44.4
ΔH°_i (kcal mol^{-1})	+12 ± 6	+12 ± 2	−2.4	−8.5	−15.4	−23.1	−30.8
ΔS°_i (kcal K^{-1} mol^{-1})	0.5 ± 0.02	0.04 ± 0.01	0.024	0.025	0.029	0.037	0.045
	TnCI						
	$i=3$	$i=3$					
ΔG°_i	−2.5 ± 0.2	−2.7 ± 0.1					
ΔH°_i	+4 ± 6	+4 ± 1					
ΔS°_i	0.02 ± 0.02	0.02 ± 0.01					

[a] For cardiac TnC, the values were calculated from the difference between TnC^- and TnC^+ from Table II. Cardiac TnCI is shown for comparsion to the cardiac TnC. Standard deviations obtained from $(\Sigma d_i)^{1/2}$ where d_i is the deviation in the thermodynamic values in Table II. [b] Data from Yamada (1978) has been converted from kJ to kcal.

one thermal transition is observed. However, the number of systems studied is too small for broad generalizations.

The thermodynamic data in the literature on troponin are scanty, and no direct comparisons can be made with our data. However, Potter et al. (1977) and Yamada (1978) have both measured Ca^{2+} binding to skeletal TnC, and some comparisons can be made by interpolation of our data on cardiac TnC. There are considerable differences between the results of Potter et al. (1977) and Yamada (1978). These differences have been attributed by Yamada (1978) to the differences in experimental conditions. Potter et al. measured $\Delta G°$, $\Delta H°$, and $\Delta S°$ at 25 °C, pH 7.0, for reaction 1 and Yamada (1978) at 10

$$TnC + xCa^{2+} \rightleftharpoons TnCCa_x \quad (1)$$

°C, pH 8.8 for reaction 2. For skeletal TnC, x could vary

$$TnC(Mg)_4 + xCa^{2+} \rightleftharpoons TnC(Mg)_{4-y}Ca_x + yMg^{2+} \quad (2)$$

between 1 and 4 since there are four possible Ca^{2+} binding sites (two Ca^{2+}–Mg^{2+} binding sites and two Ca^{2+}-specific binding sites). With Mg^{2+} present, the two Ca^{2+}–Mg^{2+} binding sites and the two Mg^{2+}-specific binding sites (Potter & Gergely, 1975) would be occupied. y would range between 1 and 2 since Ca^{2+} would displace Mg^{2+} in the two Ca^{2+}–Mg^{2+} binding sites. Both Potter et al. (1977) and Yamada (1978) found that there were two classes of Ca^{2+} binding sites in skeletal TnC and that both $\Delta G°$ and $\Delta H°$ were negative for Ca^{2+} binding while $\Delta S°$ was positive. The positive $\Delta S°$ values were attributed to changes in water by both workers.

In our work, the differences in the thermodynamic state functions between TnC^- and TnC^+ (Table II) should give the thermodynamic parameters for Ca^{2+} binding to cardiac TnC according to eq 3 since cardiac TnC has two high affinity

$$TnC(Mg)_2 + 2Ca^{2+} \rightleftharpoons TnC(Mg)_2(Ca)_2 \quad (3)$$

Ca^{2+}–Mg^{2+} binding sites, one Ca^{2+}-specific binding site, and two Mg^{2+}-specific binding sites (Leavis & Kraft, 1978; Kohama, 1979). If the TnC concentration is ~100 mg/mL, the concentration of high affinity Ca^{2+}–Mg^{2+} binding sites is ~10 mM. With 10 mM Mg^{2+} present and no Ca^{2+}, these high affinity sites should be occupied by Mg^{2+}. With 10 mM Mg^{2+} and 10 mM Ca^{2+} present, these high affinity binding sites would be occupied by Ca^{2+}, and Mg^{2+} would occupy the Mg^{2+}-specific binding sites. This equation is, of course, only an approximate representation since equilibrium exists and some occupation of low affinity sites is expected.

For cardiac TnC, the $\Delta H°$, $\Delta S°$, and $\Delta G°$ values for Ca^{2+} binding are given in Table III. Since the deviations increase as T is varied from the T_d values, the values reported here at 37 °C have smaller standard deviations and are more reliable in terms of the assumptions made in their derivation (i.e., ΔC_p^d independent of T) than the values at 25 °C. Extrapolation of our data to 10 °C for comparison to Yamada (1978) would drastically increase the standard deviations since the differences from T_d are very large. At 10 °C, the ΔG_i value would be negative but smaller than at 25 °C, and ΔH and ΔS would still be positive at 10 °C. The thermodynamic values for Ca^{2+} binding to cardiac TnC are very different than the values previously reported for skeletal TnC. ΔG is considerably smaller for the cardiac than for the skeletal TnC. This implies that binding to cardiac is less favorable than to skeletal TnC, which is in agreement with the binding constants found by Hincke et al. (1978). The most striking difference between cardiac and skeletal TnC is that the enthalpy change for the cardiac is positive, which means that the enthalpy change on binding is not favorable. In the case of the cardiac Tn, it appears that the favorable $\Delta G°$ is caused by entropy changes. The values for cardiac TnCI are also given in Table III to show that the same type of effects occur in the complex as in TnC. The concentration of TnCI in these solutions is ~2 mM. With 10 mM Ca^{2+} and 10 mM Mg^{2+} present, the high affinity Ca^{2+}–Mg^{2+} binding sites and the Ca^{2+}-specific site should be occupied by Ca^{2+}, and the Mg^{2+}-specific sites should be occupied by Mg^{2+}. The Ca^{2+} binding can be approximately represented by eq 4. This equation is only approximate since

$$TnCI(Mg)_4 + 3Ca^{2+} \rightleftharpoons TnCI(Mg)_2Ca_3 + 2Mg^{2+} \quad (4)$$

equilibrium exists and occupancy of the low affinity sites will depend in equilibrium constants. The enthalpy change in the cardiac system is also positive for the CI complex. The free enthalpy change in the cardiac system is also positive for the CI complex. The free energy changes are larger for the TnCI complex than for TnC, indicating that binding to the complex is favored.

The differences reported here on Ca^{2+} binding to cardiac TnC from previous results in the skeletal system are very likely due at least in part to intrinsic differences between cardiac and skeletal Tn. However, our experimental conditions are different from the previous measurements. The presence of Mg^{2+} and higher KCl in our sample might account for part of the differences from the results of Potter et al. (1977) at

25 °C. Part of the difference between our results and Yamada's (1978) may also be due to our higher KCl concentration. Either high salt (Mrakovčič et al., 1979) or Mg^{2+} (Kawasaki & van Eerd, 1972) can induce conformational changes. These conformational changes are similar to the conformational change caused by Ca^{2+} (van Eerd & Kawasaki, 1972). However, it has been reported that Mg^{2+} binding can cancel the salt-induced effects (Mrakovčič et al., 1979). Hence, in the presence of both high salt and Mg^{2+}, the native state of the TnC^- in this work may be quite difference than in Yamada's case. The importance of the native state in determining Ca^{2+} binding constants has already been established. The binding constants for Ca^{2+} to cardiac TnC (in absence of Mg^{2+} and in 0.15 M KCl at pH 7) obtained by Hincke et al. (1978) differ by 2–4-fold from the binding constants obtained by Stull & Buss (1977) for Ca^{2+} binding to the cardiac troponin–tropomyosin complex (with 2 mM Mg^{2+} and 0.25 M KCl at pH 7). In addition, Stull & Buss (1977) found four Ca^2 binding sites in the complex while Hincke et al. (1978) found three Ca^{2+} binding sites for cardiac TnC. Differences in T among the various workers may also contribute to the observed differences in the thermodynamic parameters. Yamada (1978) also noted that the differences between his work and Potter et al. (1977) could be explained by assuming a large negative ΔC_p for Ca^{2+} binding to TnC. We have assumed that ΔC_p^d is independent of temperature. There is no direct evidence for a large negative ΔC_p for Ca^{2+} binding or for our assumption that ΔC_p^d is independent of temperature. We observed no differences in ΔC_p^d between TnC^- and TnC^+ or in any of the troponin complexes $\pm Ca^{2+}$. However, the experimental errors in ΔC_p^d are relatively large, and small changes are possible. In conclusion, then, the differences in experimental conditions makes the direct comparison between Ca^{2+} binding to cardiac and skeletal TnC difficult, but considering the magnitude of the effects reported, it appears likely that at least some of the differences may be due to inherent differences between cardiac and skeletal TnC.

It is generally believed that the Ca^{2+}–Mg^{2+} sites are occupied by Mg^{2+} during relaxation and that the interactions with Mg^{2+} are probably involved in maintaining the native state of the Tn complex. Ca^{2+} must bind to the Ca^{2+}-specific site before contraction can occur. Since the Ca^{2+}-specific regulatory sites are of lower affinity than the Ca^{2+}–Mg^{2+} sites, Ca^{2+} probably displaces Mg^{2+} from the high affinity sites before contraction occurs. In this study of the denaturation of Tn and its subunits in the presence and absence of Ca^{2+}, the initial state was selected to be as close as possible to the in vivo state so that the Ca^{2+}–Mg^{2+} sites were occupied. The conclusion that Ca^{2+} binding is favorable when the Ca^{2+}–Mg^{2+} sites are occupied and that this Ca^{3+} binding in the cardiac system is an entropy-driven reaction is interesting in terms of the molecular mechanism of muscle contraction.

The results presented in the present study are in agreement with those obtained by using the CD melt technique (McCubbin et al., 1974; Mani et al., 1974) where it was found that the presence of Ca^{2+} had a stabilizing effect on the units and complexes. However, due to the broad melting range found in the CD melt studies, it was not possible to quantitate the stabilization conferred by Ca^{2+}.

In this work, enthalpy, the melting temperature, the entropy (for the reversible systems), and the change in heat capacity on thermal denaturation of Tn and its subunits have been measured. The importance of the mutual interaction of all three subunits in determining the properties of Tn has been established. The dominating effect of entropy changes on Ca^{2+} stabilization has been demonstrated.

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Demonstration of Nitrogen Coordination in Metal–Bleomycin Complexes by Electron Spin–Echo Envelope Spectroscopy†

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ABSTRACT: We have studied the Cu(II), Co(II), and Fe(III) complexes of the antineoplastic drug bleomycin by using electron spin–echo envelope spectroscopy. For all three complexes, nitrogen coordination of the metal ion is demonstrated. For the Cu(II)- and Co(II)–drug complexes, we have been able to identify imidazole as a metal ligand.

The bleomycins (Figure 1) are members of a family of antibiotic glycopeptides that have been isolated as Cu(II) complexes from bacterial cultures of *Streptomyces verticillus* (Umezawa et al., 1966a,b). The various bleomycins differ from each other only in their terminal functional group (Umezawa, 1979). Bleomycin breaks DNA in vitro (Suzuki et al., 1969) in a reaction requiring the O_2-dependent oxidation of drug-bound Fe(II) (Ishida & Takahashi, 1975; Sausville et al., 1976; Horwitz et al., 1979). It is believed that the in vitro antitumor activity is caused by the same reaction (Umezawa, 1979).

The structures of metal complexes of bleomycin (Muraoka et al., 1976; Sugiura, 1978) are of great interest since the in vitro activity of the drug requires the formation of an Fe(II) complex (Sausville et al., 1976, 1978b; Burger et al., 1979). Other metal ions such as Cu(II), Co(II), and Zn(II) are inactive although they inhibit the DNA–breakage reaction with Fe(II) by replacing iron in the metal–drug complex (Sausville et al., 1978a).

Structural determinations of metal–bleomycin complexes by X-ray crystallographic procedures have not met with any great success, save in a single instance (Iitaka et al., 1978), because of the difficulty of preparing samples suitable for analysis. Other physical techniques employed for structural determinations include polarography and optical, NMR, and EPR spectroscopy (Dabrowiak et al., 1978a,b; Oppenheimer et al., 1979a,b; Gupta et al., 1979; Antholine & Petering, 1979; Burger et al., 1979; Sugiura, 1979a,b; Sugiura & Ishizu, 1979; Sugiura & Mino, 1979; Sugiura et al., 1979; Solaiman et al., 1980; Lenkinski et al., 1980). Although conclusions drawn from these studies are, in many instances, inferential, they suggest that metal ions are bound to the drug via nitrogenous ligand atoms, including those from imidazole and pyrimidine.

Another physical probe, especially useful for the study of paramagnetic metalloproteins, is electron spin–echo envelope spectroscopy (Mims & Peisach, 1979a). This technique has proven to be a useful means of identifying ligands of paramagnetic metal ions and of determining the coupling between nuclei belonging to these ligands and the unpaired electron spin. In this paper, we present electron spin–echo data demonstrating unequivocably that Cu(II), Co(II), and Fe(III) are ligated to bleomycin via nitrogenous ligands. For Cu(II) and Co(II), we are able to identify imidazole as a ligand to the metal ion, based on the ^{14}N coupling frequencies. For the Fe(III) complex, the analysis is more difficult, and coupling to more than a single ^{14}N is suggested.

Materials and Methods

Bleomycin sulfate (Blenoxane) was a gift of Bristol Laboratories and contained approximately 60% bleomycin A_2, 30% bleomycin B_2, and 10% various other bleomycins. Cu(II)–BLM[1] was prepared by mixing equal volumes of 40 mM cupric acetate with 60 mM bleomycin and then diluting with an equal volume of glycerol. The pH was raised to 7.2 with concentrated NaOH. Fe(III)–BLM was prepared by using ferrous

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[1] Abbreviations used: BLM, bleomycin; TPP, tetraphenylporphyrin.

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